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MECHANISMS OF ACTION OF CLOSTRIDIAL NEUROTOXINS ON DISSOCIATED MOUSE SPINAL CORD NEURONS IN CELL CULTURE

ANNUAL REPORT

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FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council (DHHS, PHS, NIH Publication No. 86-23, Revised 1985).

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INTRODUCTION

The research conducted during this period continued the previous studies of structure-function relationships of tetanus toxin with monoclonal antibodies studied in the previous years. The focus of these studies was on utilizing the knowledge from these previous studies to provide probes for investigating the cellular mechanisms of action of the tetanus toxin.

Tetanus toxin is one of the most toxic substances known. After being bound to nerve terminals peripherally it moves by retrograde axonal transport to the ventral spinal cord (Price et al., 1975). The toxin then moves transsynaptically to the presynaptic terminals where it acts to block presynaptic release of neurotransmitter (Schwab et al., 1976, Bergey et al., 1987). Despite the extreme potency of tetanus toxin, little is known about its cellular locus of action. It has been speculated that, following binding, tetanus toxin moves intracellularly to an unknown site to ultimately block presynaptic neurotransmitter release (Schmitt et al., 1981, Critchley et al., 1985). Some recent studies with intracellular injections of tetanus toxin into adrenal chromaffin cells supports this (Penner et al., 1986).

The dissociated mouse spinal cord culture system has allowed characterization of the actions of tetanus toxin. After a dose-dependent latent period the toxin produces convulsant activity due to the preferential reduction in inhibitory synaptic transmission. This convulsant activity is characterized by paroxysmal depolarizing events (PDE), abrupt depolarizing shifts typically from 5 to 20 mV with a resulting train of action potentials, lasting 200-2000 mS before a return to resting membrane potential. The spinal cord neurons in culture are quite sensitive of tetanus toxin; as little as 0.1 mouse lethal dose (1pg/ml) is sufficient to reliably produce convulsant activity as measured by intracellular recordings. Ultimately excitatory as well as inhibitory transmission is blocked by the toxin (Bergey et al., 1981, 1983, 1987).

The tetanus toxin molecule can be cleaved to form a nonbinding fragment B and nontoxic, binding fragment C (Helting and Zwisler, 1977; see Figure 1 in Results). Recently we have been utilizing a library of monoclonal antibodies developed against various epitopes of the toxin molecule (Kenimer et al, 1983) for structure function studies of toxin action utilizing the system of dissociated mouse spinal cord in culture. Previous studies conducted in this proposal have suggested that the B fragment is the toxic subunit. A monoclonal antibody directed against the B fragment has been demonstrated to neutralize the convulsant action of the toxin while not preventing binding of the toxin.

As mentioned above, it has been speculated, and some evidence suggests that the toxin is in fact acting intracellularly. The subcellular locus, however, is not known, nor is the mechanism of action. To investigate the locus of action we experimented with a unique conjugate of the binding, nontoxic C fragment and a neutralizing monoclonal antibody directed against the toxic B subunit. The experimental hypothesis was that this conjugate could gain entry into the neurons an protect the neurons from the effects of subsequent applications of intact toxin.

MATERIALS AND METHODS

Culture Techniques

Cultures of fetal mouse spinal cord neurons were prepared as described in detail previously by Ransom et al. (1977). Spinal cords were removed from 13-14 day old fetal mice and then pretreated with trypsin before mechanical dissociation. The cells were then plated on collagen-coated 35 mm plastic culture dishes. The culture medium was Eagle's minimal essential medium (MEM) supplemented with glucose (final concentration 30 mM and bicarbonate (final concentration 44 mM). Cultures were grown and maintained at 35 C in 10% CO_2. During the first 24 hours both 10% fetal calf serum and 10% horse serum (HS) were included in the culture medium. After this time only 5% HS was included and 1% N3 solution (Romijn, 1982) was added. The antimetabolite 5-fluoro-2-deoxyuridine was used for a 24-h period after day 6 to limit the growth of nonneuronal cells. Cultures were maintained with biweekly subtotal changes of medium for 4-8 weeks at which time they were used for experiments.

Tetanus Toxin

Homogeneous tetanus toxin was prepared from sterile filtrates of Clostridium tetani cultures as previously described by Ledley et al. (1977). The toxin has about 2×10^7 mouse lethal doses (MLD) per milligram of toxin protein. An MLD is defined as the least amount of toxin that will kill a 15-18 g mouse within 96 h following subcutaneous injection into the inguinal fold region.

Monoclonal Antibody Conjugates

As discussed above in the introduction previous experiments with selected monoclonal antibodies have identified a single monoclonal antibody 21.76.10 directed against the nonbinding B subunit. Conjugates (kindly supplied by Dr. Jane Halpern, FDA) of the 21.76.10 antibody and purified fragment C (0.6 mg in phosphate buffered saline pH 7.2) were obtained following incubation with Traut's reagent (1.0mM) for 30 minutes at room temperature. Each protein was then desalted into PBS on a small G-25 column in order to remove free cross-linking reagents. After mixing the sample was chromatographed on a TSK-3000 gel filtration column in PBS. Fractions were analyzed by SDS-PAGE, and the ganglioside binding assay. SDS-PAGE revealed a high molecular weight band which was the appropriate size for an antibody-fragment C complex.

To assay (assays by J. Halpern) whether the conjugate of fragment C and the 21.76.10 antibody was bifunctional, microtiter plates were coated with 0.1 ug of GT1b in methanol and allowed to dry. The wells were blocked with PBS containing 5 mg.ml BSA. 21.76.10-Fragment C was diluted into PBS plus 1 mg/ml BSA and various dilutions were added to

Fragment C was diluted into PBS plus 1 mg/ml BSA and various dilutions were added to indivbidual wells and incubated for 2 hours. Fragment C (100 ug/ml) to block binding to tetanus toxin to GT1b plus 100,000 cpm of ^{125}I-tetanus toxin were added to each well and incubated for 1.5 hours. The plates were washed and individual wells were counted in a gamma counter.

INTACT TETANUS TCXIN

Fragment C   Fragment B   binding domain for 21.76.10 monoclonal Ab

FRAGMENT C - ANTIBODY CONJUGATE
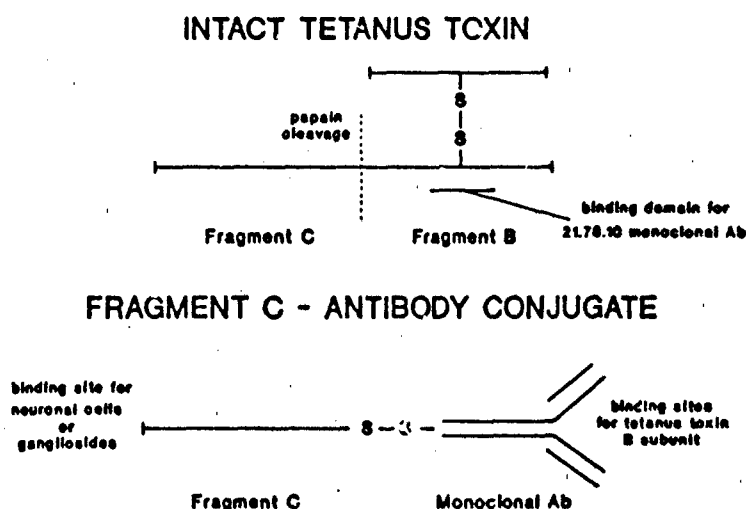

Fragment C   Monoclonal Ab

Figure 1. The native intact tetanus toxin is a two chain structure of approximately 150,000 molecular weight, comprised of a light chain (approx. 47,000 daltons) and a heavy chain (approx. 96,000 daltons). Papain cleavage can separate the toxin into a fragment C portion of the heavy chain that can bind to neurons but is nontoxic. The neutralizing monoclonal antibody 21.76.10 binds to the nonbinding region of the heavy chain of fragment B. The fragment C-antibody conjugate incorporates the monoclonal antibody and the nontoxic binding fragment C subunit.

Various experimental paridigms were used involving preincubation of the neurons with the conjugate (4 ng/ml), washes and subsequent addition of whole tetanus toxin. To allow for internalization of any conjugate that might still be cn the neuronal surface, a 6 hour period in MEM was allowed after exposure to the conjugate for 24 hrs. To further control for any possible remaining C fragment expcsed on the cell surface, some experiments included exposures to tetanus toxoid (10 ug/ml, Sclavo).

Electrophysiology

Cultures were selected for electrophysiological studies after growing for 4 to 8 weeks. Cultures were washed 3x in MEM with 1% fetal calf serum to remove all horse serum (horse serum contains antitetanus antibodies at high titers). After washing no detectable horse serum is present (i.e. <0.001 U). Cultures were then placed on a heated (33-34 C.), CO_2-gassed (10%) stage of an inverted phase-contrast microscope. Intracellular recordings were made under direct vision using microelectrodes filled with 4 M potassium acetate at neutral pH and pulled to yield resistances of 30-50 MOhm. A conventional bridge circuit was used for recordings in conjunction with a storage oscilloscope and continuous chart

recorder and storage on VHS video tape following digitalization of the signal by a pulse code modulator (A.R. Vetter).

A dilution of purified tetanus toxin (400 pg/ml) was added to the cultures in 500 ul aliquots to yield a final concentration of toxin of 100 pg/ml. In experiments using antibody preparations, excess antibody was added to stock tetanus toxin (400 pg/ml) and preincubated for 1 hour at 35 C. before adding a 500 ul aliquot to the spinal cord cultures. The 200 pg of toxin present in the 2 cc of culture media represents about 2 mouse lethal doses. Intracellular recordings were begun after the addition of the toxin preparations. Multiple neurons were sampled over the following hours to document the presence or absence of convulsant activity. Convulsant activity was manifest by the appearance of paroxysmal depolarizing events (PDE).

RESULTS

The fragment C monocolonal antibody conjugate was bifunctional as demonstrated by its ability to a) bind to ganglicside coated microtiter plates and by b) the ability of excess conjugate to reduce binding of labeled whole native toxin (Figure 2).
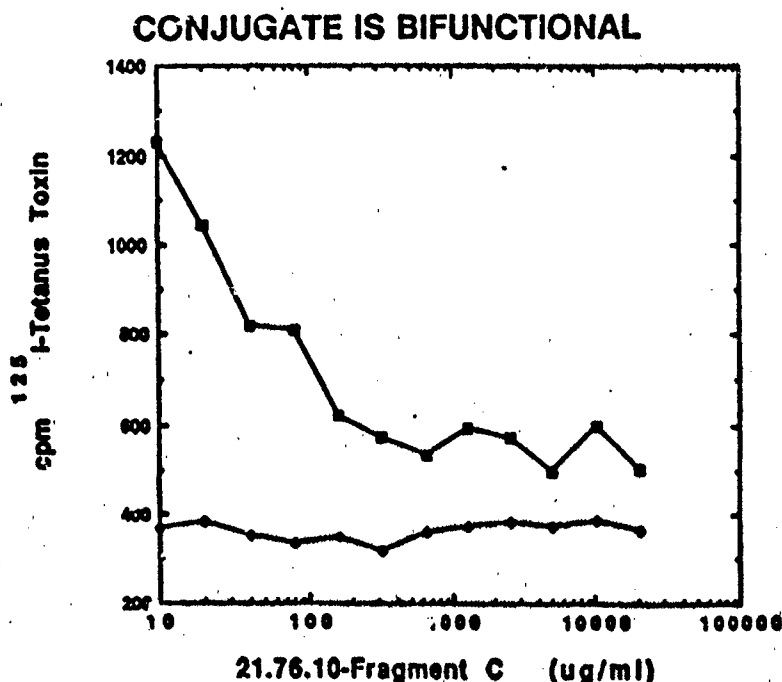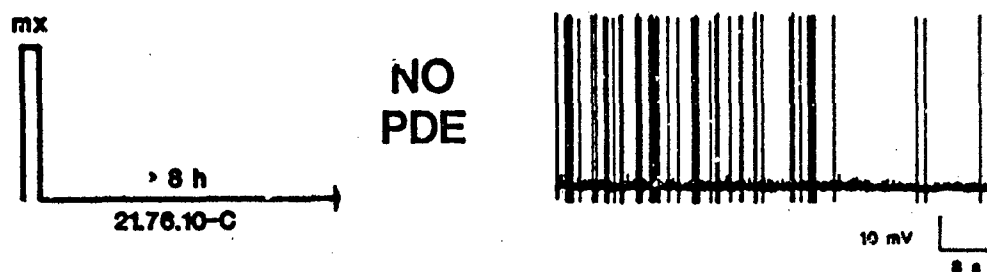

CONJUGATE IS BIFUNCTIONAL

Figure 2. The Fragment C-antibody conjugate was bifunctional. The closed circles represent background levels of intact labelled toxin binding without ganglioside coating the microtiter plates. There is no change with increasing amounts of conjugate. With ganglioside coated plates (open circles) there is increased binding of labelled toxin and preincubation of the plates with the conjugate reduces binding of whole, labelled toxin.

Addition of the fragment C-antibody conjugate alone (at concentrations of 1-4 ng/ml) produced no convulsant activity, confirming the nontoxic nature of the C fragment (C fragment alone, not shown, also does not produce convulsant activity). Addition of 100 pg/ml of tetanus toxin reliably produced paroxysmal depolarizing events after 8 hours or less (Figure 3).

FRAG. C - CONJUGATE ALONE
IS NOT CONVULSANT

mx

NO
PDE

> 8 h

21.76.10-C

10 mV

TETANUS TOXIN PRODUCES
CONVULSANT ACTION

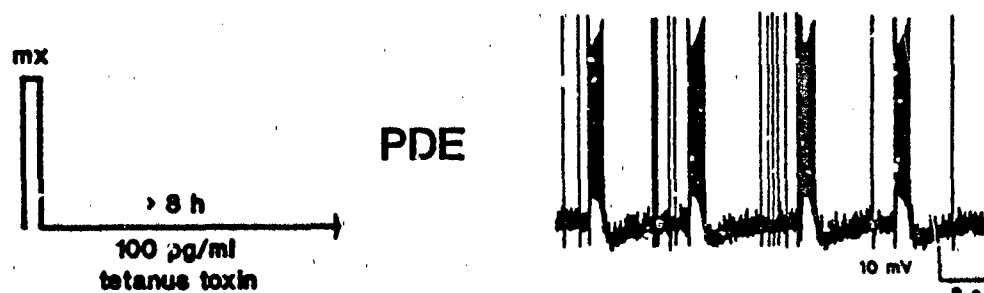mx

PDE

> 8 h

100 pg/ml
tetanus toxin

10 mV

Figure 3. The upper tracing illustrates that no convulsant activity is produced by the addition of the conjugate alone. The illustrations on the right show typical intracellular recordings from spinal cord neurons. After addition of the conjugate alone there is no change from the control pattern of in.ermixed spontaneous actions potentials and excitatory and inhibitory postsynaptic potentials. Tetanus toxin reliably produces paroxysmal depolarizing events (PDE) characterized by the bursts of action potentials shown that reflects reduced synaptic inhibition resulting from reduced presynaptic release of neurotransmitter. mx indicates media mix.

Addition of the conjugate after exposure of the spinal cord neurons to 10 or 100 pg/ml of tetanus toxin did not prevent or reverse the appearance of convulsant activity (Figure 4). Recordings were made for up to 6 days with the lower toxin concentration and the convulsant activity persisted. This toxin concentration is one of the lowest demonstrated to produce convulsant activity in these cultures.

ADDITION OF CONJUGATE
AFTER CONVULSANT ONSET
DOES NOT REVERSE TOXIN ACTION

A mx   mx

PDE

9 h   9-140 h

100 pg/ml   21.76.10-C
tetanus toxin

B mx   mx

PDE

31 h   6 d

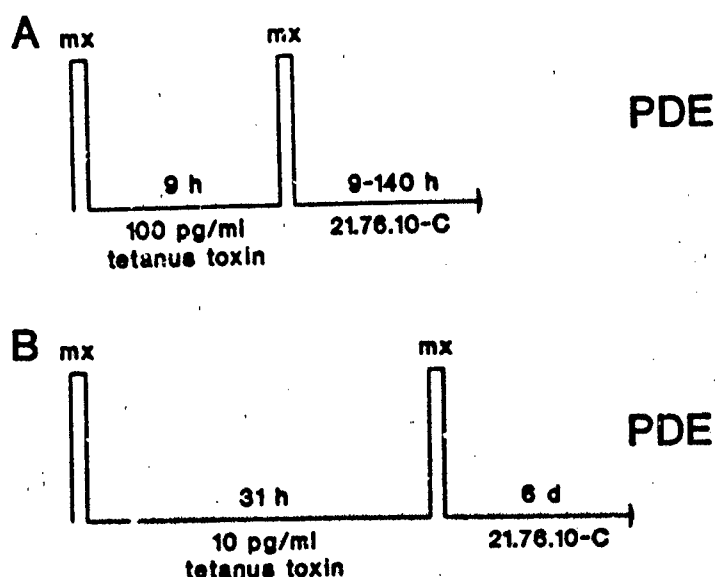10 pg/ml   21.76.10-C
tetanus toxin

Figure 4. Following preincubation of spinal cord neurons in 10 or 100 pg/ml tetanus toxin for times sufficient to produce convulsant activity, the toxin was washed off and excess fragment C-antibody conjugate was added (sufficient to neutralize the toxin). No reversal of convulsant activity was seen even after 6 days at the low toxin dose of 10 pg/mL. Convulsant activity was manifest by paroxysmal depolarizing events (PDE) as illustrated in Figure 3.

Preincubation of the spinal cord neurons with the toxin-monoclonal antibody conjugate, did provide protection against tetanus toxin action as measured by the ability of this preincubation to prevent the production of convulsant activity (Figure 5). In all instances the cells were washed prior to the addition of tetanus toxin to remove antibody conjugate not associated with the neurons. The theoretical possibility existed, however, that some of the conjugate might be cell-associated but not internalized, that is the antibody could be exposed at the cell surface and be able to neutralize the toxin prior to toxin internalization. To control for this possibility, two experimental situations were tested. In one (Figure 5B) a six hour interval was allowed prior to the addition of the tetanus toxin to allow for internalization of any bound toxin-conjugate. In other experiments (Figure 5C) a six hour interval was allowed and then the cells were incubated with excess tetanus toxoid. The toxoid does not bind to neurons, yet if any antibody were still present either in the media or on the cell surface the toxoid would be expected to bind to these sites. In all three instances the preincubation with conjugate protected against the convulsant activity of tetanus toxin.

PREINCUBATION WITH CONJUGATE
BLOCKS TOXIN EFFECT

A mx ————————————— mx

   24 h   › 8 h

   21.76.10-C   100 pg/ml
                     tetanus toxin

NO
PDE

B mx ————————————— mx ———— mx

   24 h   6 h   › 8 h

   21.76.10-C   MEM   100 pg/ml
                             tetanus toxin

NO
PDE

C mx ———————— mx ——— mx ——— mx

   24 h   6 h   6-7 h   › 24 h

   21.76.10-C   MEM   10µg/ml   100 pg/ml
                       tetanus   tetanus toxin
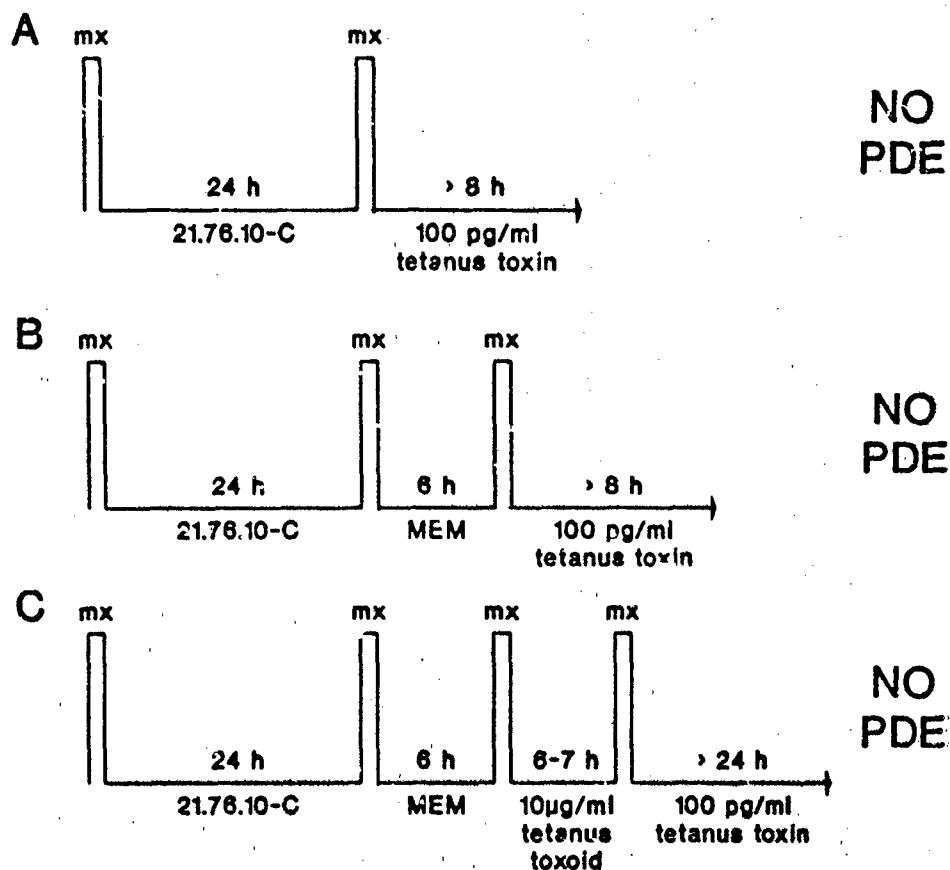   toxoid

NO
PDE

Figure 5. Preincubation of spinal cord neurons with the antibody-fragment C conjugate protected against subsequent effects of tetanus toxin. In A the toxin was added after the cells were washed (mx) in MEM X 2, in B a 6 hour incubation in MEM separated the preincubation with conjugate and the addition of toxin and in C a 6 hour incubation in MEM was followed by incubation with excess tetanus toxoid prior to washing and addition of tetanus toxin. In each instance the preincubation protected against the development of convulsant activity (PDE) produced by tetanus toxin.

DISCUSSION

The experiments above demonstrate that the nontoxic subunit of tetanus toxin (Fragment C) can be conjugated to a neutralizing monoclonal antibody directed against the nonbinding subunit (fragment D) of tetanus toxin. This fragment C-antibody complex retains

the ability to bind to gangliosides and neurons (via the C-fragment portion) and has the ability to bind to whole tetanus toxin via the monoclonal antibody portion. Previous studies have demonstrated that fragment C alone appears to be handled in a similar fashion to whole toxin, that is it binds, moves retrograde in the axons, and is transferred transsynaptically (Fishman and Carrigan, 1987, Fishman and Savitt, 1989, Evinger and Erichsen, 1986). Whether the conjugate is handled in an identical fashion is not yet determined; however, the experiments here indicate that an irreversible membrane interaction does occur and previous experiments with toxin with associated antibody suggests typcial handling (Habig et al., 1983).

Preincubation of spinal cord neurons with the fragment C-antibody complex protects the spinal cord neurons from the convulsant action of tetanus toxin. Experiments to control for any residual cell surface antibody suggest that the fragment C-antibody complex is translocated to an intracellular site where it can prevent the action of tetanus toxin. Whether the conjugate interacts with the toxin at the actual intracellular site of action or at some intermediate site remains to be determined.

Once the convulsant action of tetanus toxin has occurred, exposure to the fragment C-antibody complex cannot "rescue" the neurons, suggesting that the toxin becomes either inaccessible or its action is no longer reversible by otherwise neutralizing antibodies. These findings suggest that tetanus toxin either has been translocated to an inaccessible site or that the actual action of the toxin is irreversible. Heretofore the action of tetanus toxin has been thought to be irreversible by antibodies because the toxin was sequestered intracellularly. Intrathecal antibody fragments have been shown to pre ent transsynaptic transport of the toxin(Erdmann et al., 1981); these antibodies are not effective once the toxin has reached the presynaptic terminals. In the experiments here the irreversibility of the toxin action must be explained by another mechanism since the antibody-fragment C complex is probably internalized. The possibility that the conjugates have an irreversible membrane interaction but are not internalized does exist.

This nontoxic fragment C-antibody complex can b a valuable probe for localizing the subcellular locus of action of tetanus toxin. Conjugates of C-fragment and peroxidase have been demonstrated to localize within cytoplasmic vesicles in presynaptic terminals (Fishman and Savitt, 1989). Comparisons with whole toxin-peroxidase conjugates and experiments with the antibody conjugates, may provide further insights into the subcellular localization of tetanus toxin.

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